ATTACHMENT TO AMENDMENT AND REPLY:

APPENDIX 1

APPENDIX 1

Clinical Test Results

Materials and methods

1. Preparation of diisopropylfluorophosphate (DFP)-inactivated canine cationic trypsin

Canine cationic trypsin was purified as described previously (Waritani et al., Vet. Immunol, Immunopathol., 80: 333 to 338, 2001). Purified cationic trypsin was irreversibly inactivated with DFP (protease inhibitor). After complete inhibition, the DFP-inactivated cationic trypsin was dialyzed against 1 mM HCl, adjusted to a concentration of 100 μ g/ml using 1 mM HCl, and immediately frozen and stored at -80° C.

2. Preparation of anti-canine TLI mAb and peroxidase-conjugated Fab fractions

The mAb against canine TLI (anti-canine cationic trypsin mAb; reagent 009-303) was prepared as previously described (Waritani et al., Vet. Immunol, Immunopathol., 80: 333 to 338, 2001). The $F(ab')_2$ and Fab fractions of 009-303 were obtained by pepsin digestion followed by 2-aminoethanethiol reduction. The Fab fraction of 009-303 was conjugated with peroxidase (POD) by using N-(6-maleimidocaproyloxy)-succinimide (Ishikawa et al., J. Immunoassay 4d, 209 to 237, 1983).

3. ELISA for canine TLI

A one-step sandwich enzyme-linked immunosorbent assay (ELISA) method was developed using mAb against canine TLI (anti-canine cationic trypsin mAb; reagent 008-207, Waritani et al., Vet. Immunol, Immunopathol., 80: 333 to 338, 2001) and the POD-conjugated Fab of 009-303. The 008-207 (100μ g/ml in 0.1 M Na-phosphate buffer; pH = 7.5; 100μ l per well) was used to coat the wells of a 96-well microplate (Nalge Nunc International, USA). The plate was incubated overnight at 4° C, and then washed three times with PBS (pH = 7.0) containing 0.05% Tween-20 (TPBS). Then, the plate was blocked by adding 300 μ l, 0.1 M Na-phosphate buffer (pH = 7.0) containing 1.0% BSA to each well and incubating overnight at 4° C. The plate was washed four times with TPBS, and then 100μ l of a solution comprising 10 ng of the POD-conjugated Fab of 009-303 diluted in 0.05 M PBS (pH = 7.0) containing 1.0% BSA (PBS-BSA) was added to each well. For standards, 5μ l DFP-inactivated cationic trypsin (1 to 100 ng/ml in PBS-BSA) was added to each well; patient sample wells received 5μ l canine serum. The protein concentrations of the DFP-inactivated cationic trypsin solutions used as standards were determined by a BCA protein assay kit (Pierce, Rockford, IL, USA) using BSA as standard according to the manufacturer's instructions. The plate was incubated for 1 h at 37° C, and then washed four times with PBS. Finally, 100μ l 0.1 M citrate-phosphate buffer (pH = 4.0) containing 0.03% 2,2'-azinobis (3-ethylbenzthiazoline) sulfonic acid and 0.003% hydrogen peroxide was added to each well. The plate was incubated for 15 min at 37° C. The reaction was stopped by 1% SDS in distilled water, and the absorbance in the wells was measured by using a microplate reader (Tosoh, Japan) with filter at 405 nm/492 nm (the side of sample/reference).

4. Preparation of an ICT kit for canine TLI

The immunochromatographic test (ICT) was developed by modifying a previously described technique (Snowden and Hommel, J. Immunol. Methods, 140: 57 to 65, 1991; De Mey et al., Cell. Biol. Int. Rep., 5: 889 to 899, 1981). Briefly, 0.05 to 1μ g/ml 008-207 and 2μ g anti-mouse IgG polyclonal antibody (Bethyl Laboratories Inc., USA) were bound to a 8 mm X 25 mm nitrocellulose strip to generate sample and control lines, respectively. After blocking with 0.01 M carbonate-phosphate buffer containing 1% BSA, the strips were dried at room temperature. Next, 100 μ g 009-303 was mixed with 1 ml of gold colloid solution (British bio cell international, UK; pH = 9.0) at room temperature. After 30 min, BSA was added to 1% of the final concentration. The mixture was centrifuged at 8000 X g for 30 min at 4° C. The precipitated gold colloid-conjugated 009-303 was dissolved in 0.01 M Tris-buffered saline containing 1% BSA; this dissolved solution was absorbed into the conjugate pad, which was then dried at 58° C. The ICT kit thus comprised a nitrocellulose strip, a conjugate pad, and an absorbent pad in a plastic case (Fig. 1).

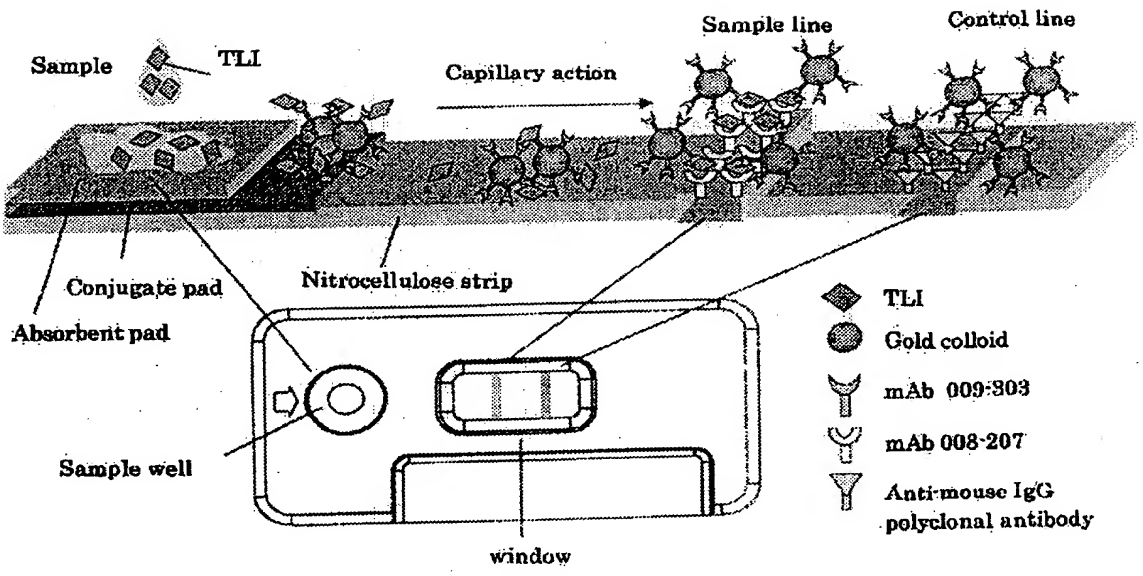

Fig. 1. Schema of the immunochromatographic test (ICT) kit.

5. Assay procedure for the ICT kit

To perform the assay, one drop of canine serum and three drops of reaction buffer (0.05 M PBS, pH = 8.0, containing 0.05% Tween-20) are added to the sample well of the case. The TLI in canine serum flows through the absorbent pad and reacts with the gold colloid-conjugated 009-303 in the conjugate pad. The immunocomplex of TLI-gold colloid-conjugated 009-303 moves by capillary action to the nitrocellulose strip. The immunocomplex reacts to the 008-207 on the sample line and is immobilized there. The excess gold colloid-conjugated 009-303 reacts to the anti-mouse IgG polyclonal antibody on the control line, and the resulting complex is immobilized there (Fig. 1). If the procedure has been carried out correctly, a red control line appears after 10 min. If a red sample line also appears (i.e. a positive result), the TLI value of the sample is > 5 ng/ml, and the dog does not have EPI. If a control line but no sample

line appears (i.e. a negative result), the TLI value of the sample is < 2 ng/ml, and the dog most likely has EPI. The canine TLI-ICT kit is produced and supplied commercially by Daiichi Fine Chemical Co., Ltd. (Takaoka, Japan).

Results

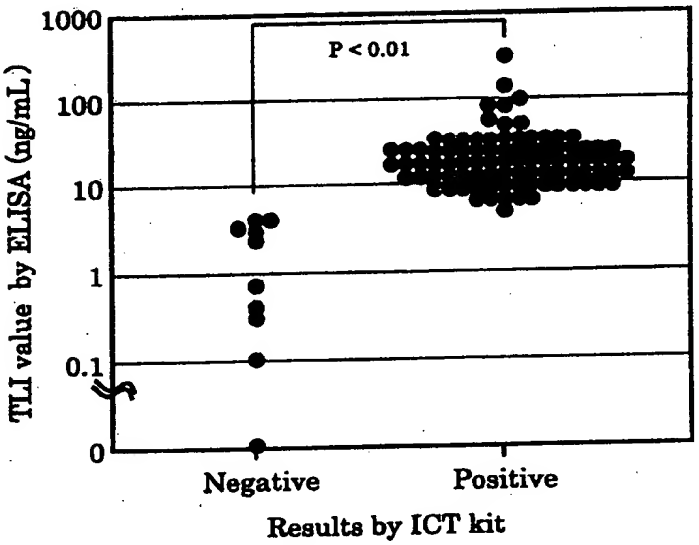

Fig. 2. Correlation between results of immunochromatographic test (ICT) kit and the ELISA-determined serum trypsin-like immunoreactivity (TLI) concentration of 65 healthy dogs and 41 dogs with exocrine pancreatic insufficiency, pancreatitis, or gastrointestinal disease.

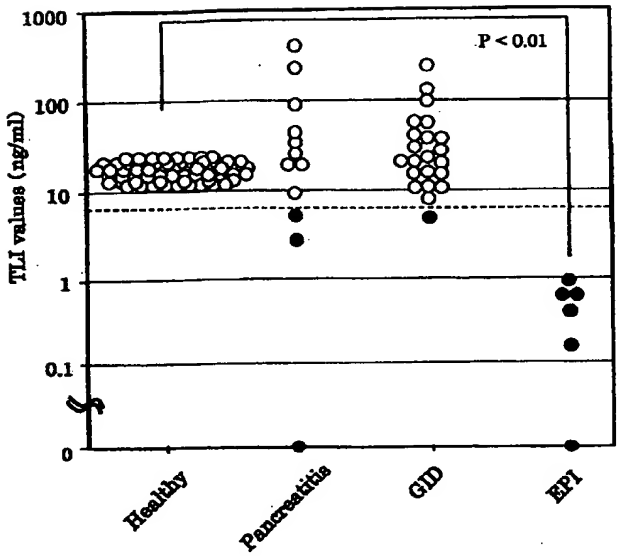

Fig. 3. ELISA-determined serum trypsin-like immunoreactivity (TLI) values for 65 healthy dogs, 12 with pancreatitis, 23 with gastrointestinal disease (GID), and six with exocrine pancreatic insufficiency (EPI). The dashed line indicates a serum TLI of 5 ng/ml, the cut-off value for the diagnosis of EPI. Positive (O) and negative (●) results from the immunochromatographic test (ICT) are from the same samples.

By ELISA, the serum TLI concentration (mean± 1 S.D.) of the 65 clinically healthy dogs was 17.0± 5.3 ng/ml (range, 7.8 to 29.2). For the 12 dogs diagnosed with pancreatitis, the average serum TLI value was 63.9± 89.8 ng/ml (range, 0.0 to 287), and that for the 23 dogs with gastroenteritis was 18.9± 18.3 ng/ml (range, 3.0 to 71.8). However, the TLI concentration was 0.3± 0.2 ng/ml (range, 0 to 0.7) in the six dogs with EPI (Fig. 3). The TLI concentrations of the clinically healthy dogs differed significantly ($P < 0.01$) from those of the animals with EPI. But TLI values did not differ between clinically healthy dogs and those with pancreatitis ($P = 0.134$) or gastroenteritis ($P = 0.825$).

From these data the TLI levels in the EPI animals are below those in the healthy dogs without exception. Accordingly, it is considered that the TLI value will serve well as a marker for EPI (Fig. 3).

From the results in connection with the canine model of pancreatitis, it is apparent that the TLI levels were elevated for 7 to 10 days after the

onset of pancreatitis and afterward returned to the extent of those values in the healthy dogs. Hence, the reason why the TLI levels in the 3 dogs with suspected pancreatitis were within the range of those values in the healthy dogs is considered that it took time after the onset. Since for the 3 dogs with low TLI levels pancreatitis turned chronic and fibrosis occurred in the pancreas, it is suggested that the pathologic transition from pancreatitis to EPI had occurred in such animals. Because TLI is an enzyme producible only in pancreas, however, inflammation can be judged to be present in pancreas (pancreatitis) when the TLI level is elevated. Therefore, it is suggested that TLI will serve as a good marker for diagnosing whether pancreatitis occurs or not.

Further, for the fact that elevated TLI levels are observed in some of the gastrointestinal disease (GID) dogs, since it is extremely difficult to diagnose decisively whether pancreatitis occurs or not and the pancreatitis is a highly lethal disease which will lead to multiorgan diseases, it is interpreted that there may be some possibility of latent pancreatitis even when other diseases are suspected. This suggests that the TLI measurement allows pancreatitis to be detected even in patients with suspected GID.

A positive ICT sample line did not occur when the TLI concentration of the canine serum sample was < 4.1 ng/ml by ELISA (Fig. 2). Further, all six dogs diagnosed with EPI were negative (no sample line) by ICT, as were three of the 12 dogs with pancreatitis and one of the 23 dogs with gastrointestinal disease (Fig. 3). From these results, ICT gives an EPI detection rate of 100% and it is clarified that EPI can be detected distinguishably from other diseases.

6. Assessment in canine model of pancreatitis (Fig. 4)

Methods and Materials

1) Establishment of canine model of pancreatitis

Healthy adult beagles were anesthetized (induction with atropine (0.05 mg/kg), Silece™ (flunitrazepam, 0.03 mg/kg) and thiopental (10 mg/kg), followed by maintenance with isoflurane) and incised mediad from chondroxiphoid tail end to umbilicus to reveal the duodenum and the pancreas. A catheter was inserted into the accessory pancreatic duct from the duodenal papilla and a trypsin-autobile mix was injected under low pressure conditions. Then the duodenum and incised wound were sutured. Blood was periodically collected prior to and after surgery. EDTA whole blood and serum samples were subjected to each test. For 2 days the animals were fasted without water and necessary hydration was conducted. From day 3, the animals were fed once a day and free of approaching to water.

2) Serochemical tests

Samples were assayed using Mono-Test α-Amylase EPS (Roche) for amylase and Rikitekku Lipase, Color (Roche) for lipase, with COBAS-MIR <Plus> (Roche) according to protocols attached to the analyzer. The samples were also assayed for TLI by ELISA.

Results

Three dogs clinically diagnosed as being healthy were used to establish pancreatitis models. Mean values and standard deviations of serochemical test data were chronologically plotted. In all the experimentally acute pancreatitis dogs, levels of amylase, lipase and TLI were elevated from hour 1 after the induction, then decreased once on day 3 or 4 though they were higher than those in healthy dogs, afterward reelevated and the high levels retained until days 7 to 10. Since TLI is especially increased more rapidly and significantly than amylase and lipase, it is recognized that it will serve as a marker sensitive to pancreatitis.

Discussion

The TLI values were elevated in all of three experimentally acute pancreatitis dogs after the pancreatitis induction. Therefore, it is judged that the TLI value is useful as a marker for pancreatitis. However, the TLI was elevated only for about 7 to 10 days and afterward showed a tendency to return to the range of those in the healthy animals. Accordingly, this suggests: the results that TLI is not always elevated even in pancreatitis are attributable to the measurement conducted in patients wherein the disease has passed over a certain period after the onset but not to failures in the increase of TLI.

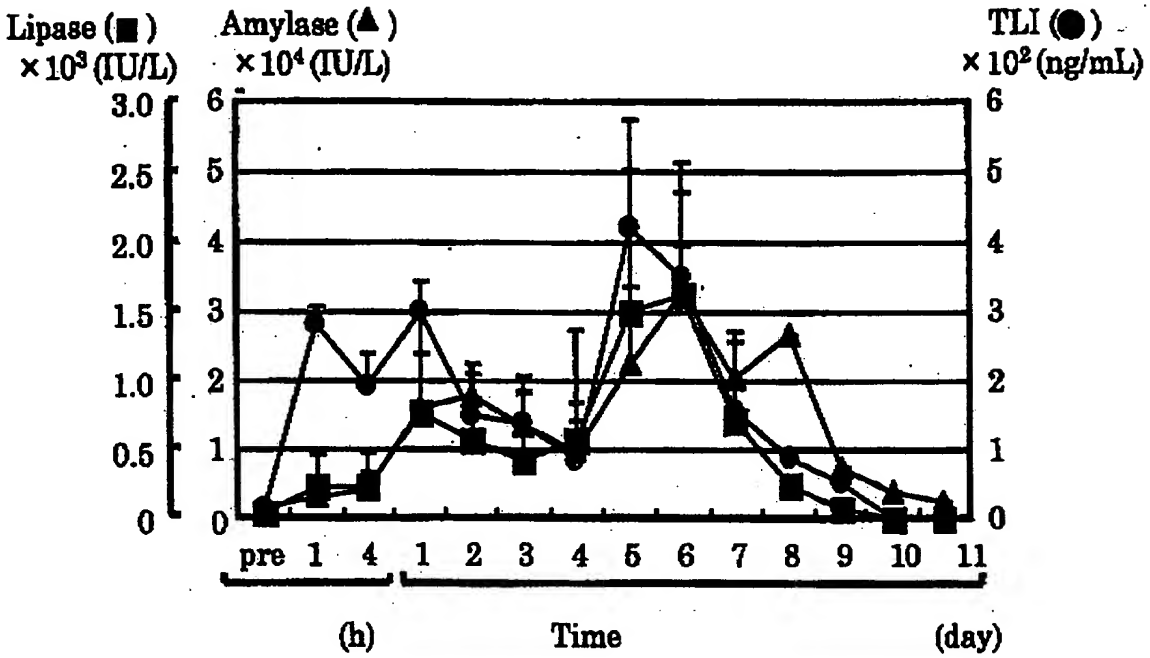

Fig.4 The time course of TLI , amylase or lipase values in experimentally acute pancreatitis dogs induced mixture of autobile and trypsin in material and methods.

7. Assessment for ELISA Specificity (Fig. 5)

Methods and Materials

Preparation of trypsin complexed with proteinase inhibitor

Purified canine cationic trypsin was mixed in combination with human α_1-antitrypsin (SIGMA) at a molar ratio of 10.8 in 0.1M Tris-HCl (pH 8.0) containing 25mM $CaCl_2$. Similarly, the purified canine cationic trypsin was mixed in combination with human α_2-macroglobulin (SIGMA) at a molar ratio of 6 in 0.1M Tris-HCl (pH 8.0) containing 25mM $CaCl_2$. The resultant mixtures were incubated at room temperature for 1 hour.

Inhibition of the proteinase inhibitor against trypsin was checked by assaying the activity with BAEE. Assay samples were diluted with PBS containing 1% BSA by referring to the concentration of trypsin.
ELISA for assaying TLI was performed in the same manner as aforementioned.

Results

The ELISA failed to measure bovine, porcine and canine anionic trypsins. Canine cationic trypsin complexed with α_2-macroglobulin was nonmeasurable within the range of ordinary assay levels (1 to 100 ng/mL) with the ELISA.

In contrast, canine cationic trypsin complexed with α_1-antitrypsin was measurable though an approximately 30% decrease in assay sensitivity was observed.

Discussion

Since the ELISA cannot measure bovine and porcine trypsins, it is judged to be highly specific. Although there are some cases where bovine pancreatic enzymes (e.g., pancreatin) and other drugs are generally administered as aids for digestion in the treatment of canine exocrine pancreatic insufficiency, the ELISA is not affected adversely for TLI assays even if bovine trypsin is absorbed and transferred into blood.

Further, since it cannot measure canine anionic trypsin which has 80% homology to canine cationic trypsin in view of amino acid levels, it is considered to be highly specific. Canine cationic trypsin complexed with α_1-antitrypsin is measurable though observed values reduce at a rate of approximately 30%. It is uncovered that the TLI value obtained by the ELISA is the total of the canine cationic trypsin and the canine cationic trypsin complexed with α_1-antitrypsin. However, the ELISA failed to measure canine cationic trypsin complexed with α_2-macroglobulin within ranges for ordinary assays. Since α_2-macroglobulin is a macromolecule with an extremely large size (molecular weight: about 800 kDa), it is interpretable that trypsin will be embedded in the construct upon its combination therewith so as to reduce its reactivity with an epitope of mAb.

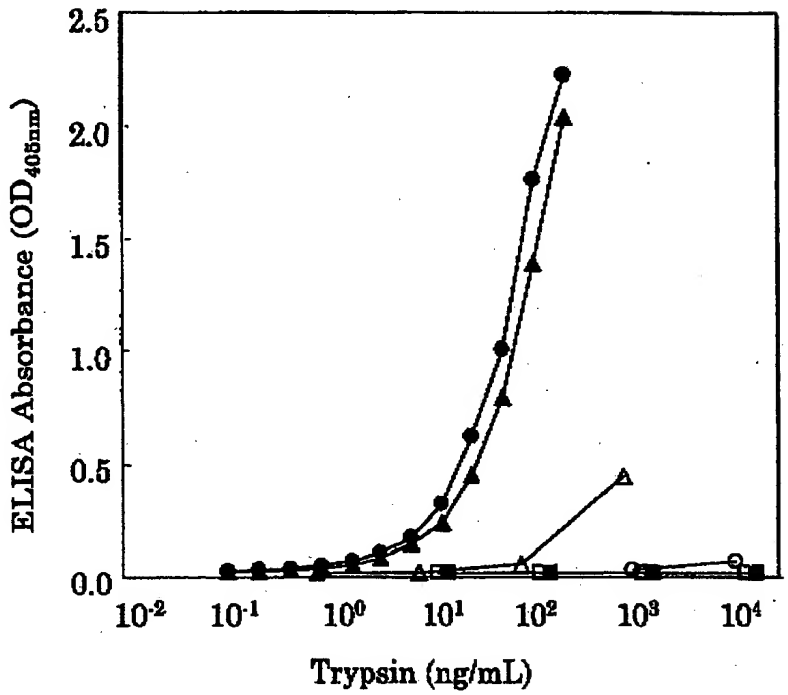

Fig.5 Cross-reactivity of mAb on ELISA with trypsin complex, canine cationic, canine anionic, bovine and porcine trypsins. The x-axis represents trypsin concentrations determined by the protein assay kit for sample of trypsin and trypsin complexes. ●,canine cationic trypsin ; ○,canine anionic trypsin; ▲,canine cationic trypsin complexed with α_1-antitrypsin; △,canine cationic trypsin complexed with α_2-macroglobulin ; ■,bovine tryopsin ; □, porcine trypsin.